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DETERMINATION AND CHARACTERIZATION OF SELECTED FLUOROQUINOLONES OXIDATION PRODUCTS UNDER POTASSIUM PERMANGANATE TREATMENT

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Abstract: A simple, sensitive and reproducible ultra-performance liquid chromatography (UPLC) method for the determination of: danofloxacin, enrofloxacin, marbofloxacin, orbifloxacin and pefloxacin oxidation stability under permanganate treatment in acidic conditions at pH from 3.0 to 6.0, was developed. Chromatographic separations were carried out using the Acquity UPLC BEH C_{18} column; (2.1 × 100 mm, 1.7 µm particle size). The column was maintained at 40°C, and eluted under gradient conditions using from 100% to 75% of eluent A over 10 min for danofloxacin, enrofloxacin, marbofloxacin, orbifloxacin or 95% to 75% of eluent A over 10 min for pefloxacin, at a flow rate of 0.3 mL/min. Eluent A: 1% (v/v) formic acid in water; eluent B: 0.1% (v/v) formic acid in acetonitrile. Satisfactory resolution was obtained for oxidation products. The correlation coefficients and determination coefficients (R^2) obtained for linear model for all examined fluoroquinolones were greater than 0.99. Linearity range was observed in the concentration range 0.06–0.13 mg/mL. Sensitivity of the method was good. The LOD and LOQ values were found to be 0.01 mg/mL and from 0.02 to 0.04 mg/mL, respectively. Good precision and intermediate precision with %RSD less than 2.0% was observed. The oxidation processes followed kinetic of the second order reaction and depended upon solution acidity. Oxidation of fluoroquinolones proceeded at piperazine moiety yielding respective hydroxy and oxo analogs, and leaving the quinolone fragment intact. Structures of products formed were assigned on a basis of UPLC/MS/MS fragmentation pathways.

Keywords: fluoroquinolones, oxidation, kinetic evaluation, UPLC-MS/MS

Fluoroquinolones are antimicrobial agents widely used in human and veterinary medicine due to their broad activity spectrum against Gram-positive and Gram-negative bacteria and good oral intake properties. After administration, fluoroquinolones are only partially metabolized in the body and largely excreted in their pharmacologically active forms (1, 2).

In stability studies of pharmaceuticals, beside hydrolysis in acidic and basic solutions and verification of photostability, evaluation of an influence of oxidizing agents has been recommended (3). It is worth noting, that the oxidation process of drugs, especially antibiotics, has focused more attention as an arising issue in the environmental protection.

The literature survey evidenced results of oxidation of ciprofloxacin, enrofloxacin, lomefloxacin, norfloxacin, ofloxacin, pipemidic acid and flumequine using MnO_2 followed by evaluation of the reaction kinetics and analysis of chemical structure of degradation products formed (4, 5).

Moxifloxacin, as well as ciprofloxacin, difloxacin, lomefloxacin, norfloxacin and ofloxacin and theirs oxidation products during reaction with $KMnO_4$ in acidic solution of pH ranging from 3.0 to 6.0 were determined by UPLC-MS/MS. Mechanism of the process, the kinetic analysis and structures of oxidation products were presented in our recent publications (6, 7).

Moreover, an advanced oxidation technologies, notably ozonation of thirteen fluoroquinolone, including danofloxacin, enrofloxacin, pefloxacin and sparfloxacin with a secondary wastewater effluent matrix was also reported (8).

Similar studies were carried out for ciprofloxacin, enrofloxacin and flumequine in phosphate buffer solutions at pH values ranging from 3.0 to 9.0. Moiety-specific reaction kinetics were meas-

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ured and elimination of biological activity during aqueous ozonation processes was investigated (9, 10).

Another advanced oxidation process, electron pulse radiolysis, was studied for chemical degradation of danofloxacin, enrofloxacin, marbofloxacin, orbifloxacin and flumequine in buffer solution adjusted to pH 7.0. Kinetics and degradation mechanism were investigated. In addition, the products of γ irradiation degradation of fluoroquinolones were analyzed by LC-MS to elucidate the probable pathways of advanced oxidation/reduction processes (AO/RPs) degradation (11).

Liu et al. studied the degradation products of danofloxacin under stressed conditions (hydrolysis, oxidation and photolysis). The oxidative study was carried out in 30% H_2O_2 at room temperature for 12 h. Seven degradation products were detected using hybrid ion trap/time-of-flight mass spectrometry (LC/MS-IT-TOF) (12).

Other advanced oxidation processes, catalytic wet air oxidation, anodic oxidation with electrogenerated H_2O_2, electro-Fenton, photoelectron-Fenton and solar photoelectron-Fenton, oxidation with H_2O_2 in the presence of copper oxide, titanium carbide and silicon nitride nanoparticles as well as oxidation by chlorine dioxide and conductive-diamond electrochemical oxidation, were studied for the chemical degradation of enrofloxacin in aqueous solution (13-17). The primary degradation products formed during the process have been identified (13-15).

Recently our interest focused on UPLC coupled with mass spectrometry technique for the separation and identification of oxidation products since the efficiency, sensitivity and run time became an important factor in the pharmaceutical analysis.

Herein, we report on the development of a new UPLC-MS/MS method for the determination of danofloxacin (DAN), enrofloxacin (ENR), marbofloxacin (MAR), orbifloxacin (ORB), pefloxacin (PEF) and their oxidation products during reaction with $KMnO_4$ in solutions of different pH ranging from 3.0 to 6.0. The method was used for kinetic studies and identification of obtained degradation products of examined fluoroquinolones.

Potassium permanganate is widely used as an oxidizing agent in synthetic as well as in analytical chemistry and also as a disinfectant. Permanganate Mn(VII) is the most potent oxidation state in alkaline as well as in acid medium. The oxidation by permanganate ion finds extensive application in organic syntheses, especially since the advent of phase transfer catalysis. Kinetic studies are important sources of mechanistic information on the reaction.

The presence and accumulation of fluoroquinolones in aquatic environments even at low concentrations may pose threats to the ecosystem and human health by inducing increase and spread of bacteria drug-resistance due to long-term exposure. Chemical oxidation using permanganate has been widely used for treatment of pollutants in drinking water and wastewater applications for over 50 years (18, 19).

In view of potential pharmaceutical importance of DAN, ENR, MAR, ORB and PEF and lack of the literature on this type of oxidation of examined drugs and the complexity of the reaction, a detailed study of the reaction becomes important. Our study deals with the title reaction to investigate the redox chemistry of permanganate in solutions of different pH and to arrive at a suitable mechanism for oxidation of DAN, ENR, MAR, ORB and PEF by permanganate on the basis of kinetic results. It seems, that oxidation of model fluoroquinolones under $KMnO_4$ in acidic medium, opens up the possibility for application in drug stability studies and environmental protection studies in the process of utilization of drug traces.

EXPERIMENTAL

Chemicals and reagents

Danofloxacin Cat. No. 33700-100MG-R Sigma-Aldrich (Germany), Enrofloxacin Cat. No. OR6-237 Ranbaxy (Poland), Marbofloxacin CRS, European Pharmacopoeia Reference Standard, Council of Europe – EDQM CS 30026 F-67081 (France, Strasbourg, Cedex), Orbifloxacin Cat. No. 34041-100MG-R Sigma-Aldrich (Germany), Pefloxacin mesylate dihydrate Cat. No. P0106-10G Sigma-Aldrich (Germany). HPLC grade methanol, acetonitrile and formic acid (98%) were purchased from J.T. Baker (Netherlands). HPLC grade water was obtained from HLP 5 (HYDROLAB Poland) apparatus and was filtered through 0.2 μm filter before use.

Standard solution

The amount of 0.2 g of all fluoroquinolones was weighed with a precision of 0.1 mg. PEF was dissolved in the volume of 50 mL of methanol, and filled up to 100 mL with the same solvent. The sample weight of DAN and ORB was dissolved in the volume of 5 mL of glacial acetic acid, and filled up to 100 mL with methanol. The sample weight of ENR was dissolved in 48 mL of methanol with the addition of 2 mL of chloroform, and filled up to 100 mL with the same solvent. Solution of MAR was

prepared by dissolving the amount of 0.2 g of MAR in the volume of 50 mL of methanol-water mixture (1 : 1, v/v), and filled up to 100 mL with the same solvent. For method validation, solutions containing different concentrations of the examined fluoroquinolones in the range 0.06–0.13 mg/mL were prepared.

Oxidation study of the drug substance

The amount of 0.5 mL of methanol solutions of DAN, ENR, MAR, ORB, PEF (2.0 mg/mL), 2.5 mL demineralized water, 5.0 mL ammonium acetate buffer solution prepared according to European Pharmacopeia (16) with proper pH (3.0, 4.0, 4.5, 5.0 or 6.0) and 2 mL 0.002 M $KMnO_4$ was added to 10.0 mL flasks. The test solutions were incubated at room temperature and 1 µL of each reaction mixture was injected onto ACQUITY UPLC system after 15, 30, 45 and 60 min, respectively. Before the measurements of test samples, the analysis of solutions containing identical components as test samples but without $KMnO_4$ was done. The analyses were performed in triplicate.

UPLC/MS/MS analysis

The UPLC-MS/MS system consisted of a Waters ACQUITY® UPLC® (Waters Corporation, Milford, MA, USA) coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole). Chromatographic separations were carried out using the Acquity UPLC BEH (bridged ethyl hybrid) C_{18} column; 2.1 × 100 mm, and 1.7 µm particle size. The column was maintained at 40°C, and eluted under gradient conditions using from 100% to 75% of eluent A over 10 min for DAN, ENR MAR, ORB or 95% to 75% of eluent A over 10 min for PEF, at a flow rate of 0.3 mL/min. Eluent A: 1% (v/v) formic acid in water; eluent B: 0.1% (v/v) formic acid in acetonitrile.

Chromatograms were recorded using Waters eλ PDA detector. Compound concentration (%*i*) after oxidation induced by $KMnO_4$ was calculated from quotient of peak area (A*i*) to the sum of all peaks areas (Σ*A*) on chromatograms according to formulation $\%i = (Ai/\Sigma A)100$ at $\lambda = 294$ nm. Spectra were analyzed in 200-700 nm range with 1.2 nm resolution and sampling rate 20 points/s.

MS detection settings of Waters TQD mass spectrometer were as follows: source temperature 150°C, desolvation temperature 350°C, desolvation gas flow rate 600 L/h, cone gas flow 100 L/h, capillary potential 3.00 kV, cone potential 20 V. Nitrogen was used for both nebulizing and drying gas. The data were obtained in a scan mode ranging from 50 to 1000 m/z in time 0.5 s intervals; 8 scans were summed up to get the final spectrum.

Collision activated dissociations (CAD) analyses were carried out with the energy of 30 eV, and all the fragmentations were observed in the source. Consequently, the ion spectra were obtained by scanning from 50 to 500 m/z range. Data acquisition software was MassLynx V 4.1 (Waters).

Method validation

The described method was validated for the determination of DAN, ENR, MAR, ORB and PEF in the presence of oxidation products by UPLC method according to ICH guidelines (20, 21).

Specificity

To demonstrate the specificity of the developed UPLC method the solutions of DAN, ENR, MAR, ORB, PEF after oxidation stress were analyzed. Oxidation study was performed in 0.002 M $KMnO_4$ solution in ammonium acetate buffer at pH 4.0; the solution was left for 15 min at room temperature.

System suitability

The system suitability parameters were defined with respect to tailing factor and resolution of examined fluoroquinolones peaks using solutions of DAN, ENR, MAR, ORB, PEF, after oxidation stress at pH 4.0.

Linearity

The linearity for DAN, ENR, MAR, ORB, PEF was assessed by injecting six separately prepared solutions covering the range of 0.06–0.13 mg/mL. The slope of regression line, y-intercept, standard deviation of slope and intercept, correlation coefficient, R^2 value and standard error of residuals of the calibration curve were calculated using the program Statistica v. 10. Next, to determine whether the residuals have normal distribution, the Shapiro-Wilk statistical test was used.

Limit of detection (LOD) and limit of quantification (LOQ)

Based on the standard error of residuals (Se) and the slope (a) of the calibration plots and following the formula LOD = 3.3Se/a and LOQ = 10Se/a, the LOD and LOQ values for chosen fluoroquinolones were estimated.

Precision

The repeatability of the method was checked by a sixfold analysis of the concentration level 0.10

Table 1. Validation of the method.

Parameter	DAN	ENR	MAR	ORB	PEF
t_R (min)[a]	7.19 ± 0.05	7.49 ± 0.03	6.21 ± 0.02	7.64 ± 0.06	5.37 ± 0.04
Resolution[b]	6.00	5.50	1.33	6.40	7.00
LOD (mg/mL)	0.01	0.01	0.01	0.01	0.01
LOQ (mg/mL)	0.03	0.02	0.02	0.04	0.03
Linear range (mg/mL)	0.06 – 0.12	0.07 – 0.13	0.06 – 0.12	0.06 – 0.13	0.06 – 0.13
Regression equation (y):					
Slope ($a \pm S_a$)	189794.7 ± 5336.2	210743.2 ± 4944.8	346248.3 ± 6511.1	35454.2 ± 12962.1	116615.0 ± 3335.4
Intercept ($b \pm S_b$)	–183.2 ± 491.5	–1160.2 ± 526.3	–1245.4 ± 582.4.5	–1453.7 ± 1287.9	–200.5 ± 331.4
$t = b/S_b$	$-0.37 < t_{\alpha,f}$ statistically insignificant	$-2.20 < t_{\alpha,f}$ statistically insignificant	$-2.14 < t_{\alpha,f}$ statistically insignificant	$-1.13 < t_{\alpha,f}$ statistically insignificant	$-0.61 < t_{\alpha,f}$ statistically insignificant
Shapiro-Wilk test for residuals	0.8747 (p = 0.07)	0.9568 (p = 0.75)	0.9157 (p = 0.32)	0.9273 (p = 0.35)	0.9128 (p = 0.23)
Correlation coefficient	0.9996	0.9978	0.9986	0.9934	0.9959
R^2 value	0.9989	0.9951	0.9968	0.9855	0.9911
Precision (% RSD)	0.56	0.89	0.51	0.82	0.48
Intermediate precision (% RSD)	1.10	1.21	0.93	1.18	1.01

$t_{\alpha,f} = 2.306$ critical value of Student's t-test for degrees of freedom f = 8 and significance level $\alpha = 0.05$. [a] Mean ± SD (n = 6), [b] The mean values for n = 6. Resolutions were calculated between two adjacent peaks.

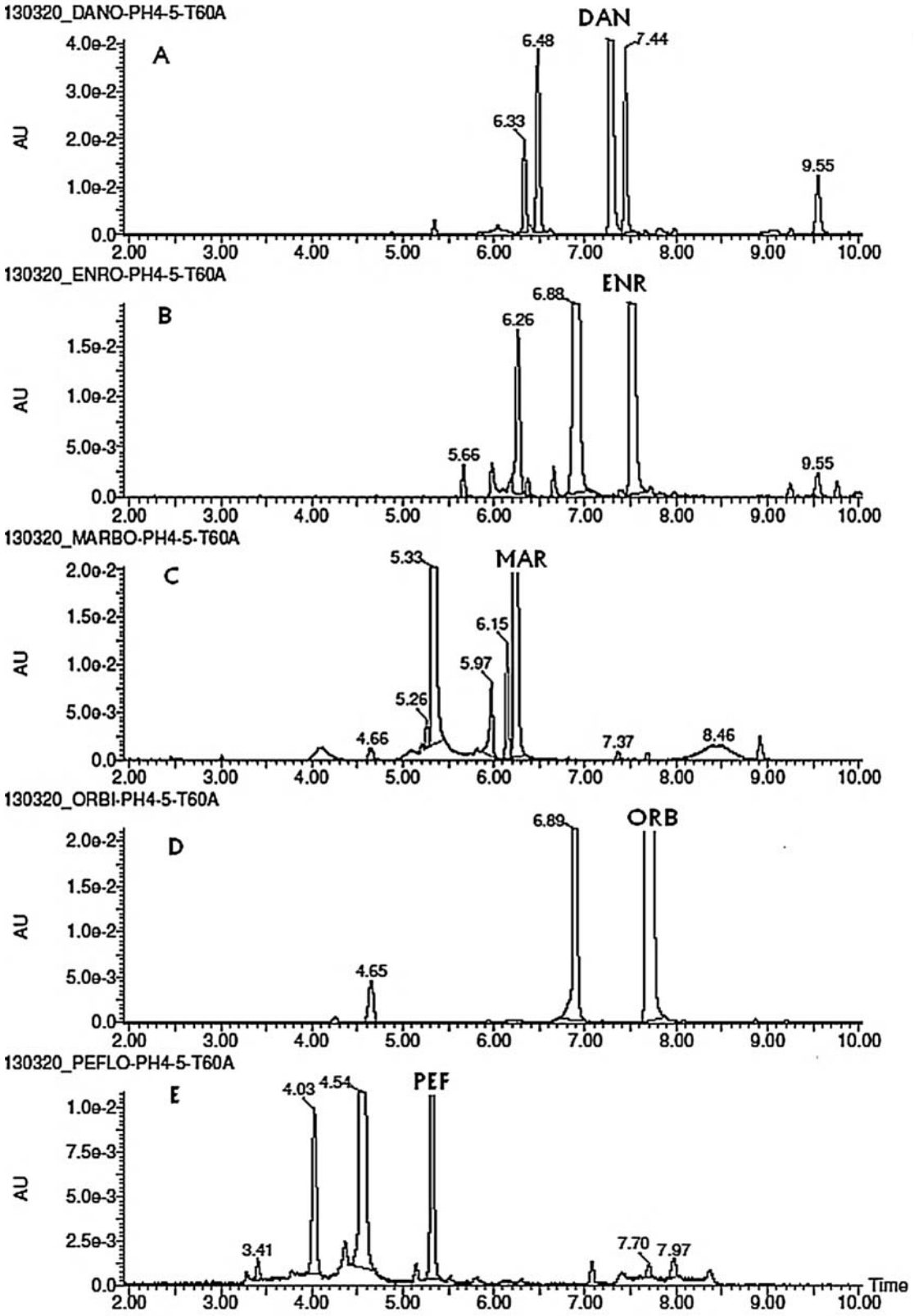


Figure 1. UPLC chromatograms registered for fluoroquinolones after oxidation induced by $KMnO_4$ at pH = 4.5: A, DAN t_R = 7.24; B, ENR t_R = 7.50; C, MAR t_R = 6.23; D, ORB t_R = 7.68; E, PEF t_R = 5.33

mg/mL of DAN, ENR, MAR, ORB, PEF solutions. The same protocol was followed for three different days to study the intermediate precision of the proposed method. Different analysts prepared different solutions on different days. The RSD (%) of the peak area of examined fluoroquinolones was calculated.

Robustness

To demonstrate the robustness of the method deliberate small changes of flow rate, content of acetonitrile and column temperature were made around the optimal values. The mobile phase flow rate was 0.30 mL/min; to study the effect of the flow rate on resolution, the flow rate was changed to 0.27 and 0.33 mL/min. The effect of the column temperature was studied at 36°C and 44°C (instead of 40°C), and the mobile phase composition was changed +5% from the initial composition.

RESULTS AND DISCUSSION

Apart from hydrolysis and photostability assays, an influence of oxidizing agents in the process of forced degradation is an integral part of

Table 2. Oxidation reaction rate constant of fluoroquinolones in solution at different pH.

Component	pH	Rate constant k [min^{-1}]	Correlation coefficient r
PEF	3.0	3.00×10^{-4}	0.9908
	4.0	11.00×10^{-4}	0.9596
	5.0	11.00×10^{-4}	0.9760
	6.0	3.00×10^{-4}	0.9994
MAR	3.0	3.00×10^{-4}	0.9531
	4.0	3.00×10^{-4}	0.9852
	5.0	3.00×10^{-4}	0.9892
	6.0	1.00×10^{-4}	0.9833
ENR	3.0	0.65×10^{-4}	0.9998
	4.0	2.00×10^{-4}	0.9967
	5.0	2.00×10^{-4}	0.9955
	6.0	0.98×10^{-4}	0.9837
DAN	3.0	2.00×10^{-4}	0.9242
	4.0	1.00×10^{-4}	0.8537
	5.0	2.00×10^{-4}	0.8771
	6.0	1.00×10^{-4}	0.8303
ORB	3.0	0.35×10^{-4}	0.9949
	4.0	0.32×10^{-4}	0.9678
	5.0	0.27×10^{-4}	0.9806
	6.0	0.22×10^{-4}	0.9774

Table 3. The kinetic results of investigated fluoroquinolones under $KMnO_4$ oxidation at pH 4.5 at room temperature.

Component	Rate constant k [min^{-1}]	$t_{0.1}$ [min]	$t_{0.5}$ [min]	Correlation coefficient r
DAN	2.00×10^{-4}	5.56	50.00	0.9169
ENR	2.00×10^{-4}	5.56	50.00	0.9788
MAR	3.00×10^{-4}	3.70	33.33	0.9937
ORB	0.25×10^{-4}	44.44	400.00	0.9709
PEF	10.00×10^{-4}	1.11	10.00	0.9654

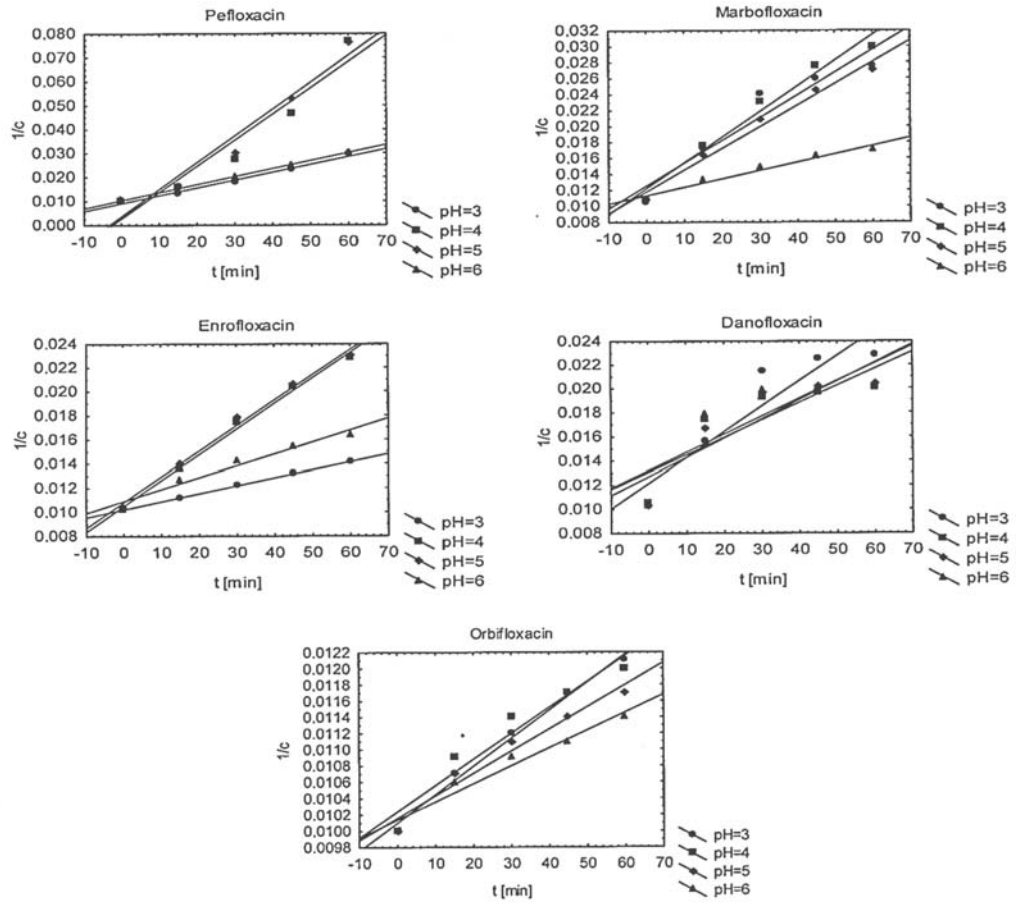


Figure 2. The 1/c = f(t) graph of oxidation of DAN, ENR, MAR, ORB and PEF induced by $KMnO_4$ in acidic medium

stress studies used for the stability evaluation of pharmaceutical products and should be checked (3). This effect may be evaluated by using validated analytical procedures enabling the determination of decreasing concentration of examined substance and detection of degradation products.

Optimization of chromatographic conditions

The main target of the chromatographic method was to achieve the separation of oxidation products and the main components DAN, ENR, MAR, ORB and PEF. To optimize the chromatographic separation, preliminary experiments were performed to test mobile phases containing different mixtures of acetonitrile and water (50/50, 83/17, 75/25, v/v); always the same amount of mobile phase additive was used. The solutions of determined fluoroquinolones after oxidation stress (buffer solution at pH = 4.0, 30 min incubation) was analyzed. We took advantage of a mixture acetonitrile/water 83/17 (v/v), with 0.1% of formic acid to obtain good peak resolution and symmetry.

Method validation

The developed UPLC method was specific to examined fluoroquinolones and guaranteed obtaining well shaped peaks both for active substances and coexisting oxidation products. Peaks of main components were well resolved from oxidation products in chromatograms and no interference that could have an influence on the obtained results was possible (Table 1). The main peak purity was examined with MS spectra using CODA algorithm (Waters Corporation, Milford, MA, USA). The investigated MS spectra uniquely contained signals corresponding to the chosen fluoroquinolones and solvent.

Satisfactory resolution was also obtained for oxidation products, peaks appearing in chromatograms were sufficiently well resolved and could be analyzed by mass spectrometry (Fig. 1).

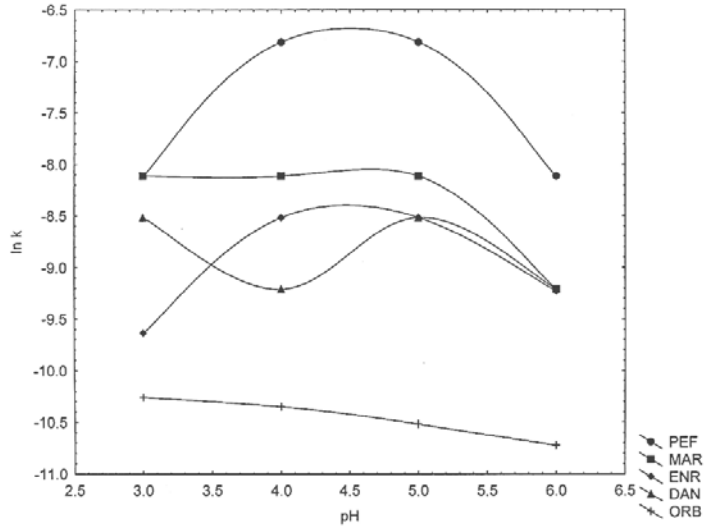
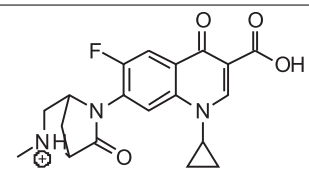
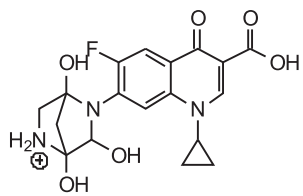
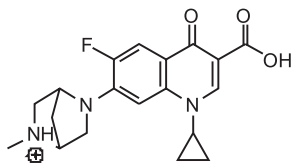
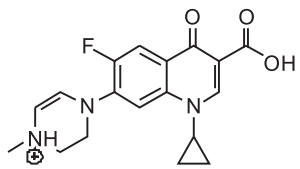
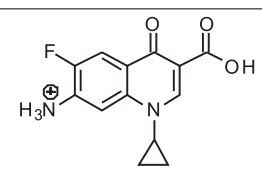


Figure 3. The ln k = f (pH) graph of oxidation of DAN, ENR, MAR, ORB and PEF induced by $KMnO_4$

Table 4. Products of oxidation of DAN induced by $KMnO_4$ in acidic conditions.

Product	RT [min]	$[M + H]^+$	Fragmentation ions	Proposed structure
DP-1	6.33	372.1	354.1, 328.2, 300.2, 284.1, 257.1, 245.1, 219.1	
DP-2	6.48	392.1	374.1, 356.1, 330.1, 312.1, 269.1, 257.1, 245.1, 219.1	
DAN	7.24	358.2	340.2, 314.2, 283.1, 271.1, 269.1, 257.1, 255.1, 245.1, 231.1, 229.1, 219.1	
DP-3	7.44	344.1	326.1, 300.2, 284.1, 257.1, 245.1, 219.1	
DP-4	9.55	263.1	245.1, 219.1, 205.0	

Regression analysis results obtained for examined fluoroquinolones are presented in Table 1. The correlation coefficients and determination coefficients (R^2) obtained for linear model for all determined fluoroquinolones were greater than 0.99. The y-intercepts of the linear equation for DAN, ENR, MAR, ORB and PEF were statistically insignificant. The distribution of the residuals can well be approximated with a normal distribution as it is shown by p-values ($p > 0.05$) of the Shapiro-Wilk normality test. Based on regression analysis, it was assumed that the calibration data fitted well to linear model. Linearity range, was observed in the concentration range 0.06–0.13 mg/mL for examined fluoroquinolones.

Sensitivity of the method was good. The LOD and LOQ values were found to be 0.01 mg/mL and from 0.02 to 0.04 mg/mL, respectively. Good precision and intermediate precision with %RSD less than 2.0% was observed. Detailed results are presented in Table 1. In all the deliberately varied chro-

Table 5. Products of oxidation of ENR induced by $KMnO_4$ in acidic conditions.

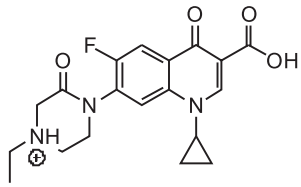
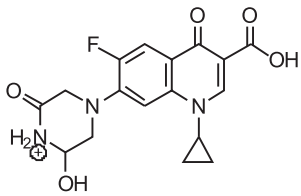
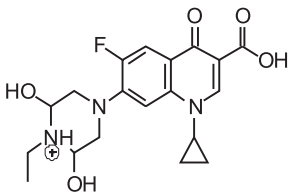
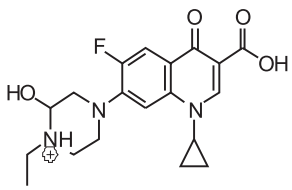
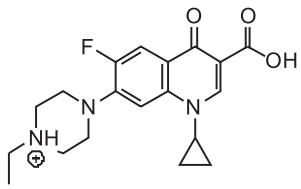
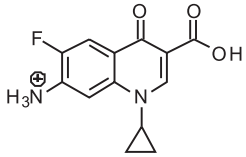
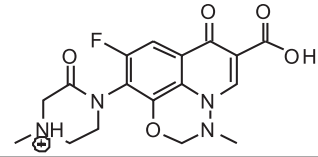
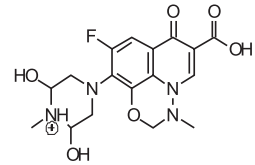
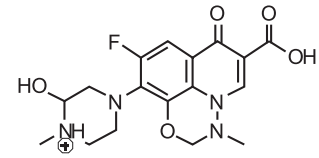
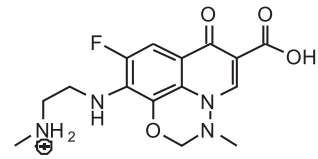
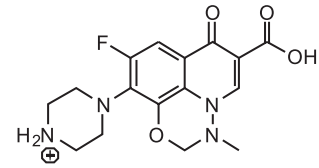
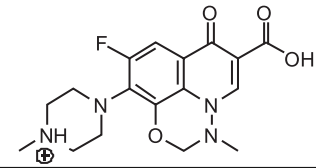
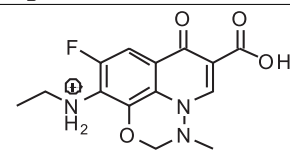
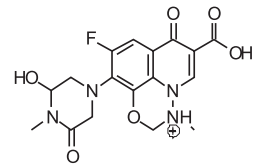
Product	RT [min]	$[M + H]^+$	Fragmentation ions	Proposed structure
EP-1	5.66	374.2	356.1, 346.1, 330.2, 328.1, 302.1, 287.1, 259.1, 245.1, 219.1	
EP-2	6.26	362.1	362.1, 344.1, 318.1, 290.1, 272.1, 245.1, 217.1, 191.1	
EP-3	6.37	392.2	374.2, 356.1, 330.2, 312.2, 284.1, 271.1, 245.1	
EP-4	6.88	376.2	358.2, 314.2, 286.1, 258.1, 230.1	
ENR	7.45	360.2	342.2, 316.2, 288.2, 286.2, 260.1, 258.1, 245.1, 217.1, 205.1	
EP-5	9.55	263.1	245.1, 219.1, 205.0, 179.1	

Table 6. Products of oxidation of MAR induced by $KMnO_4$ in acidic conditions.

Product	RT [min]	$[M + H]^+$	Fragmentation ions	Proposed structure
MP-1	4.66	377.1	359.1, 349.1, 333.1, 305.1, 289.1, 262.1, 246.1, 218.1, 192.1	
MP-2	5.26	395.1	377.1, 351.2, 337.1, 333.1, 315.1, 309.1, 279.1, 262.1, 236.1, 218.1	
MP-3	5.33	379.1	361.1, 343.1, 317.1, 275.1, 247.1, 233.1, 194.1	
MP-4	5.97	337.1	319.1, 303.1, 293.1, 264.1, 262.1, 250.1, 234.1, 206.1	
MP-5	6.15	349.1	331.1, 305.1, 288.1, 231.1, 191.1	
MAR	6.19	363.2	345.1, 319.2, 276.1, 260.1, 248.1, 234.1, 219.1, 205.1, 191.1, 163.1	
MP-6	7.37	308.1	290.1, 276.1, 250.1, 222.1, 207.1, 194.1	
MP-7	8.46	393.1	375.1, 289.1, 275.1, 218.1, 151.1	

matographic conditions (flow rate, column temperature, mobile phase composition), chosen fluoroquinolones and degradation products were adequately resolved, and the order of elution remained unchanged.

Oxidation of examined fluoroquinolones by $KMnO_4$ in acidic medium

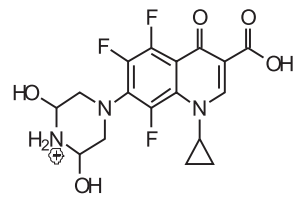
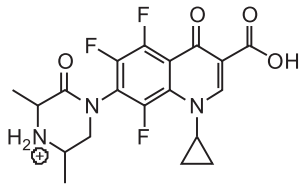
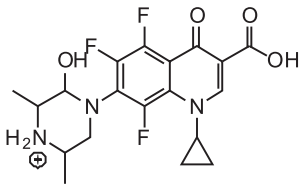
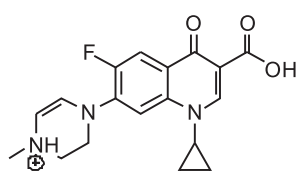
The effect of $KMnO_4$ on the oxidation of DAN, ENR, MAR, ORB and PEF has been tested in solutions of pH 3.0 to 6.0. It was found that the oxida-

tion process is dependent on the pH of the solution, incubation time and the type of fluoroquinolone. Immediately after mixing the solutions, only a single peak of fluoroquinolones deriving from starting materials were observed in chromatograms of the tested solutions. During the oxidation process, additional peaks appeared in chromatograms, whose area increased with increasing reaction time. After 60 min of incubation, the ORB had three oxidation products (OP-1 - OP-3), DAN four oxidation products (DP-1 - DP-4), and in the case of ENR and PEF five oxidation products were observed (EP-1 - EP-5 and PP-1 - PP-5). The highest number of the oxidation products was obtained for MAR (MP-1 - MP-7). Some chosen chromatograms presented in Figure 1 show the degradation profile of DAN, ENR, MAR, PEF and ORB. Degradation process of tested fluoroquinolones increased with increasing acidity of the solution in the pH range from 6.0 to 3.0, generally reaching the highest values at pH 4.5. The percentage of degradation products in the final stage of oxidation after 60 min was as follows: 48.92% for DAN, 59.51% for ENR, 62.74% for MAR, 13.5% for ORB and 85.33% for PEF.

Kinetic evaluation

The analysis of the equation $1 / c = f (t)$ for the oxidation of DAN, ENR, MAR, ORB and PEF in the pH range 3.0-6.0 demonstrated that oxidation process followed the kinetics of second order reaction (Fig. 2). Calculated kinetic parameters for the oxidation reaction for individual fluoroquinolones suggest that the oxidation process is dependent on the pH of the reaction medium and the type of fluoroquinolone tested (Table 2). In solutions at pH = 4.5 PEF oxidizes quickly – $k = 10.00 \times 10^{-4}$/min, DAN and ENR – $k = 2.00 \times 10^{-4}$/min in similar way but slower than MAR – $k = 3.00 \times 10^{-4}$/min, while the slowest oxidation was observed for ORB – $k = 0.25 \times 10^{-4}$/min. The oxidation processes described by values $t_{0.1}$ and $t_{0.5}$ determining the time after which the concentration of the tested fluoroquinolone decreases, show that oxidation will proceed in the following order PEF > MAR > DAN =

Table 7. Products of oxidation of ORB induced by $KMnO_4$ in acidic conditions.

Product	RT [min]	$[M + H]^+$	Fragmentation ions	Proposed structure
OP-1	4.65	400.1	356.1, 328.1, 310.1, 292.1, 268.1, 241.1, 227.1	
OP-2	6.20	410.1	392.1, 382.1, 366.1, 364.1, 338.2, 298.1, 227.1	
OP-3	6.90	412.2	394.1, 350.2, 334.1, 308.1, 267.1	
ORB	7.44	344.1	378.1, 352.2, 336.1, 335.1, 321.1, 307.1, 295.1, 267.1, 255.1, 227.1	

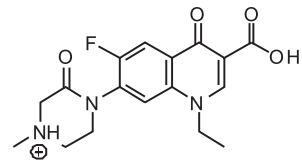
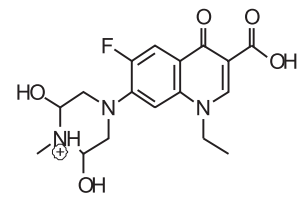
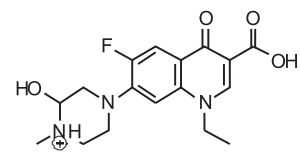
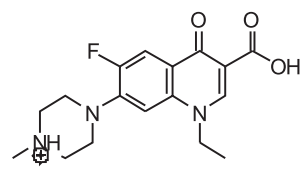
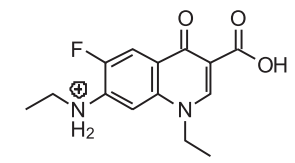
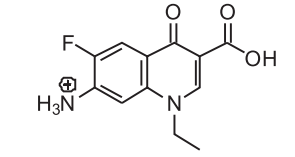
ENR > ORB (Table 3). Results of these studies confirm the degradation profiles, described by the equation ln k = f (pH), which are different and dependent on the pH of the solutions of the individual compounds and the type of examined fluoroquinolone from the smallest ORB to the largest PEF (Fig. 3).

Identification of oxidation products

The identification of oxidation products of five fluoroquinolones (DAN, ENR, MAR, ORB and PEF) induced by $KMnO_4$ in acidic conditions was performed on a basis of UPLC/MS analysis and supported by fragmentation patterns obtained from MS/MS experiments. As we have reported in our earlier paper (7), the oxidation process mainly affect the 7-amine substituent of fluoroquinolone moiety, i.e., 5-ethyl-2,5-diazabicyclo[2.2.1]heptane, 4-ethylpiperazine, 4-methylpiperazine and 3,5-dimethylpiperazine, while the fluoroquinolone core remains unchanged.

It was found that the main route of oxidation involved hydroxylation in the close vicinity of *N*-1 and

Table 8. Products of oxidation of PEF induced by $KMnO_4$ in acidic conditions.

Product	RT [min]	$[M + H]^+$	Fragmentation ions	Proposed structure
PP-1	3.41	348.1	330.1, 302.1, 276.2, 245.1, 233.1	
PP-2	4.03	366.2	348.1, 330.1, 312.1, 286.1, 260.1, 232.1, 218.1	
PP-3	4.54	350.2	332.1, 288.2, 277.1, 260.1, 245.1, 231.1, 217.1, 207.1	
PEF	5.26	334.2	316.2, 290.2, 233.1, 205.1	
PP-4	7.70	279.1	261.1, 235.1, 207.1, 179.1, 151.1	
PP-5	7.97	251.1	233.1, 207.1, 179.1	

N-4 piperazine atoms to respective hydroxylated derivatives and subsequent oxidation to their oxo-counterparts. For ENR, MAR and PEF, the main product of oxidation was 3-hydroxy derivative (EP-4, MP-3, PP-3), and 3,5-dihydroxy derivatives were also observed (EP-3, MP-2, PP-2). In case of ENR and MAR, the hydroxylactam derivatives (EP-2, MP-7), as products of further oxidation of piperazine moiety, were observed, although for ENR the intermediate step was dealkylation of *N*-4 nitrogen atom. For DAN the most abundant product of oxidation was *N*-4 demethylated 2,3,5-trihydroxy derivative (DP-2), mono-hydroxy or di-hydroxy derivatives were not observed. In case of ORB, possessing piperazine moiety with 3 and 5 position substituted with methyl group, the main product of oxidation was 2-hydroxy derivative (OP-3). Oxidation of piperazine moiety of ORB in close vicinity to more basic *N*-4 nitrogen atom, leading to 3,5-dihydroxy derivative (OP-1), involved demethylation by oxidative C-C bond cleavage, and was 10-fold less effective than oxidation in position 2. Oxidative C-C bond cleavage occurred also in case of DAN, leading to 1,2,3,4-tetrahydropyrazine derivative (DP-3). Further oxidation of fluoroquinolones led to ring opening, dealkylation (e.g., MP-4, MP-5, MP-6, PP-4) and finally yielded 7-amino quinolone products (DP-4, EP-5, PP-5). This product was not observed in case of MAR and OFL, probably due to the presence of electron-withdrawing substituents in both *ortho* positions of phenyl ring, i.e., fluorine and oxygen atoms in case of MAR and two fluorine atoms in case of OFL, and hence lower basicity of *N*-1 nitrogen atom of piperazine moiety than in case of the other investigated fluoroquinolones.

The structures of presented stable oxidation products were confirmed by Collisionally Activated Decomposition (CAD) experiments. The fragmentation pattern involved loss of H_2O from the carboxylate group of quinolone, then loss of carboxylate function and piperazine ring degradation. Quinolone *N*-1 nitrogen dealkylation proceeded slowly, except for MAR, for which 3,4-dihydro-2*H*-1,3,4-oxadiazine ring easily underwent degradation processes. For ORB, cyclopropyl moiety seems to be stable, what may be attributed to an influence of electron-withdrawing of additional fluorine substituent in position 8 of quinolone. Proposed structures of oxidation products of the examined fluoroquinolones are presented in Tables 4-8, for DAN, ENR, MAR, ORB, and PEF, respectively.

CONCLUSIONS

The elaborated method complies with the acceptance criteria for methods which can be useful for the determination of DAN, ENR, MAR, ORB and PEF in the presence of its oxidation products. The method was completely validated showing satisfactory data for all the parameters tested. Executed studies have shown that an oxidation process followed kinetics of the second order reaction for the substrates. Kinetic parameters such as rate constants k and the times $t_{0.1}$ and $t_{0.5}$ depended on solution acidity and type of studied fluoroquinolones.

It was found, that the most susceptible fluoroquinolones for oxidation was PEF. DAN, ENR and MAR displayed lower degradation rate with comparable $t_{0.1}$ and $t_{0.5}$ values, while ORB was the most stable among the tested drugs. The proposed model of investigation of oxidation profile for presented fluoroquinolones may be regarded as a predictor of their stability.

Degradation of fluoroquinolones mainly affected piperazine moiety giving respective hydroxy- and oxo-derivatives. Further oxidation of DAN, ENR, MAR and PEF, following dealkylation, and degradation of piperazine yielded 7-amino fluroquionolones analogs.

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